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Effects of ericoid mycorrhizal fungi on reproductive traits in Vaccinium corymbosum

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EFFECTS OF ERICOID MYCORRHIZAL FUNGI ON REPRODUCTIVE TRAITS IN *VACCINIUM CORYMBOSUM*

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ABSTRACT

Most angiosperms rely on animal pollination to reproduce and the majority of these also interact with mycorrhizal fungi. Although these interactions have been studied separately, few studies have examined their combined effects on host plants. Linking above and belowground interactions has become an exciting new field of study.

Ericoid mycorrhizae (ericoids) are the relationship between certain taxa of fungi and plants in the Ericaceae, including *Vaccinium corymbosum*, the highbush blueberry. Here, I asked whether inoculation with ericoid mycorrhizal fungi altered resource allocation to floral buds and flowers of *V. corymbosum*. Different fungi may vary in their ability to assist their plant partners with nutrient uptake and to address this, I inoculated plants with either a commercial or local fungal inoculum.

Inoculation with ericoids may change the number of *V. corymbosum* buds and flowers and/or affect floral traits, by enhancing nutrient uptake. If the floral traits that are affected are important to pollinators, mycorrhizae could indirectly affect the host plant's interaction with pollinators.

I hypothesized that inoculation with ericoid mycorrhizal fungi increases seed set in *V. corymbosum*, through its effects on floral traits and pollinator visitation, and responds more strongly to a local soil inoculum than to a commercial inoculum. To test my hypothesis, I inoculated 380, two-year old *V. corymbosum* plants in the spring of 2018 and randomly assigned them to one of five treatments: 1) commercial inoculum, in a peat base 2) local soil, 3) commercial inoculum and local soil, 4) a control group with no inoculum, and 5) peat base used for the commercial inoculum. Plants were then grown in a common garden.

In the summer of 2019, I transported plants to blueberry farms known to differ in pollinator abundance and conducted pollinator observations throughout the flowering season. In addition, I conducted hand-pollination experiments to examine the degree of pollen limitation at each of these farms. My results show that inoculation with ericoids directly enhanced the chances of plants flowering but did not alter interactions with pollinators. My results elucidate the importance of ericoids for the development of reproductive traits.

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1.1. Introduction

Approximately 80% of angiosperms require animal pollinators to successfully reproduce (Ollerton et al. 2011) and, of these, more than 85% simultaneously interact with mycorrhizal fungi (Brundrett 2009). Although each of these interactions has been extensively studied independently, their combined effects on plant hosts have received much less attention. Linking above and belowground interactions such as these has become a new frontier in ecological, evolutionary, and agricultural research (Gange and Smith 2005, Becklin et al. 2011, Brody et al. 2019), as their combined effects are critical to a complete understanding of a species' ecology and evolution, as well as potentially important to crop production or yield.

The association of plants with mycorrhizal fungi dates back ca. 400 million years (Pirozynski and Dalpe 1989) making it one of the oldest, most stable, and most essential symbioses in the world. Mycorrhizal fungi are thought to have helped plants transition from aquatic to terrestrial systems (Pirozynski 1981) by assisting plants with nutrient acquisition. This relationship still exists and is vital for ca. 90% of extant land plants. Mycorrhizae (the symbiosis between plant roots and mycorrhizal fungi) are now known to increase plant nutrient uptake (Stribley et al. 1975), enhance defense against soil pathogens (Perrin 1990), and improve the ability of plants to withstand

environmental stresses such as drought (Reid 1979).

Although mycorrhizae are usually beneficial, both plant and fungal genotypes vary in their quality as partners. Experiments with potted plants have shown that plants perform better when inoculated with soil from their native range rather than inocula from other habitats (Taheri and Bever 2010, Middleton et al. 2015). Several studies report better plant growth when inoculated with native soil inocula when compared to commercial inocula (Rowe et al. 2007, Paluch et al. 2013).

Inoculation with commercial and local strains of fungi may alter flower production but results up to this point have been inconsistent. For example, inoculation with commercial mycorrhizal fungi increased flower production in *Medicago truncatula* (Liu et al. 2018), *Antirhinum majus* (Asrar et al. 2012), and in *Vaccinium corymbosum*, but only for some cultivars (Brody et al. 2019). Neither local nor commercial inoculum increased flower production in *Salvia columbariae* (Aprahamian et al. 2016). Native mycorrhizal inoculum decreased flower production in Cucumis sativus in comparison to the commercial inoculum (Barber et al. 2013). In one study, flower production increased in plants with naturally occurring mycorrhizal fungi when compared to plants treated with fungicide to eliminate or reduce the presence of mycorrhizae, but only under certain ecological conditions, such as when leaf litter was present (Bennett and Cahill 2018). Inoculation by commercial inoculum

increased fruit production in *Abutilon theophrasti* (Lu and Koide 1994) and *Antirrhinum majus* (Asrar et al. 2012) but not in *Fragaria ananassa* (Niemi and Vestberg 1992). Overall, results are inconsistent and differ between plant-fungal partners.

Mycorrhizal fungi can enhance traits important to pollinators (Gange and Smith 2005) and several studies have connected the interactions of mycorrhizal fungi to floral traits, reproductive success and pollinator behavior (Cahill et al. 2008, Becklin et al. 2011, Barber and Gorden 2015). However, again, the results have been inconsistent. In a native grassland, all insect-pollinated plants with mycorrhizae had more floral visitors than the fungicide treated plants, which had lower levels of mycorrhizal colonization (Bennett and Cahill 2018). Fungicide application, to reduce mycorrhizal colonization, on a field containing wildflowers caused an overall shift in the identity of the floral visitors and had species-specific effects on floral visitor rates (Cahill et al. 2008). For example, fungicide application increased pollinator visitation to *Cerastium arvense*, and decreased pollinator visitation to *Aster laevis* (Cahill et al. 2008).

An increase in fruit production may be directly related to the contribution of the mycorrhizal inoculum to plant resource status, but could also be due to an indirect effect of mycorrhizal fungi on the interaction between plants and their pollinators. If inoculated plants receive more floral visitors because they produce more flowers or floral rewards, for example, then fruit set (the percentage of flowers that produce

fruits) could increase. For fruit set to increase as a function of pollinator activity, mycorrhizal fungi would need to alter specific plant traits that are important to pollinator behavior.

Flower size, number, and flowering phenology can affect the behavior of pollinators and, ultimately, reproductive success. Plants with larger floral displays may outcompete plants with smaller floral displays for pollinators (Bell et al. 2005). Additionally, floral phenology has been shown to affect the number of floral visitors and the identity of those floral visitors. For example, flowers that bloomed earlier in the season in *Vaccinium hirtum* were visited by fewer pollinators than later blooming flowers (Mahoro 2002). In *V. corymbosum*, the flowers that bloomed at an intermediate time point were visited by more *Apis* bees than the earliest or latest flowers of the season (Daly et al. 2013).

The abundance of pollinators in an area may also affect which plants receive effective pollination. Levels of pollen limitation can differ based on pollinator abundance and pollinator identity (Javorek et al. 2002, Cusser et al. 2016, Garibaldi et al. 2016). The effects of floral traits on pollen limitation may vary under different pollinator contexts (Totland 2001). For example, in an area with few pollinators, it is likely that specific floral traits could affect whether or not a flower is pollinated. However, in an area that has a larger abundance of pollinators, it is less likely that specific floral traits have an effect of whether or not a flower is pollinated.

Many studies linking mycorrhizae and aboveground interactions focus on plants that form arbuscular mycorrhizae (Gange and Smith 2005, Cahill et al. 2008, Becklin et al. 2011) or orchid mycorrhizae (Waterman and Bidartondo 2008, Waterman et al. 2011). There has been significantly less research done on ericoid mycorrhizae (Brody et al. 2019). Ericoid mycorrhizae form between certain fungal taxa (mostly Ascomycota) and plants within the Ericaceae family. This symbiosis evolved much later than arbuscular mycorrhizae, ca. 40 million years ago, and is thought to allow Ericaceous plants to live in harsh environments (Cairney and Meharg 2003) and specifically increase a host plant's nitrogen uptake (Kerley and Read 1998). This symbiosis may affect a plant's investment in its reproductive structures, such as flowers (Brody et al. 2019).

Inoculation with ericoid mycorrhizal fungi may alter inflorescence buds, flowers or fruits of *V. corymbosum*. If ericoid mycorrhizal fungi affect floral traits in *V. corymbosum*, it is also possible that the interaction between *V. corymbosum* and its floral visitors could also be altered. *Vaccinium corymbosum* can be pollen limited (Nicholson and Ricketts 2019) and to address this, I included different locations as a study variable. I hypothesized that inoculation with ericoid mycorrhizal fungi increases reproductive fitness in *V. corymbosum*, through its effects on floral traits and pollinator visitation. Moreover, I hypothesized that plants would benefit more from inoculation

with local soils than to commercial inoculum due to the local soil being more adapted to the Vermont climate. Specifically, I asked: does the inoculation of *V. corymbosum* with commercial inoculum or local soil inoculum containing ericoid mycorrhizal fungi 1) increase colonization of *V. corymbosum* roots? 2) alter the number or size of reproductive structures? 3) influence timing of bloom start or peak floral bloom? 4) alter interactions between *V. corymbosum* and its floral visitors in areas with different levels pollinator diversity and abundance? 5) increase fruit traits and yield?

1.2. Methods

To examine if floral and flowering traits respond to inoculation of *V. corymbosum* with ericoid mycorrhizal fungi, I conducted the following experiments. In March of 2018, 380, 2-year old *Vaccinium corymbosum* cv. Bluecrop plants were obtained from Hartmann's Plant Company, Lacota, Michigan, USA. Plants were randomly assigned to one of five treatments: 1) inoculated with commercial ericoid inoculum (Plant HealthTM) which includes spores of *Hymenoscyphus ericae* and *Oidiodendrum griseum*, (N = 90), 2) inoculated with soil from a local farm taken from the rhizosphere of blueberry plants (N=90), 3) a combination of the first two treatments (N = 90), 4) a peat control that is the base used in the commercial inoculum (N = 20), and 5) a non-inoculated control (N = 90). Plants were removed from their pot, the soil washed from the roots, and the remaining root ball covered with ca. 6 oz of inoculum, soil, or peat, which was applied by hand to the wet roots before placing them in a 7-gallon pot filled with a customized potting mix that was 12:6:3:1 peat:compost:perlite:vermiculite. Compost was purchased from Green Mountain Compost in Williston, VT. Compost consisted of leaf and yard waste and food scraps from the Champlain Valley, wood chips, a small amount of horse manure, and high carbon wood ash. Plants were then placed into 10 x 9 arrays, with the exception of the peat base control treatment place in a 10 x 2 array, at the UVM Horticulture Farm, grown for the remainder of the summer, and then overwintered by digging individual holes into the ground, placing them in the ground in their pots, and covering them

with straw mulch. Plants were fertilized before fruiting each year with 10 mL of fertilizer per pot which was based on the recommended amount of 400 L per acre of SUPERthrive fertilizer with an NPK ratio of 4:1:1.

To examine whether inoculation increased colonization by ericoids, roots were collected, stained, and scored for fungal structures, twice each year throughout the experiment. For each collection (April and September 2018, and June and September 2019), roots were collected from 15 plants in each treatment that had been sampled in the previous collection plus an additional 15, previously unsampled, plants. Small roots were collected from the edge of the root mass in four quadrants of the pot. Roots were placed in Ziploc bags, kept on ice, and transported to the laboratory where they were stored in the refrigerator until processed.

Within 48 hours of collection, all roots were washed and cleaned of excess soil and stored in 80% ethanol until staining. To begin the staining procedure, roots were sandwiched between pieces of nylon in histology cassettes and added to a flask with 10% KOH. They were autoclaved for 45 minutes at 121°C, rinsed with distilled water 3 times, and then treated with H2O2 for 20 minutes at room temperature. Finally, they were rinsed and treated with a 5% acetic acid and ink (v/v) stain and heated in a water bath at 85°C for 24 hours. Roots were rinsed with distilled water for 20 minutes and stored in distilled water at 4°C until scoring. Roots were scored at 400X for fungal structures using methods in (McGonigle et al. 1990). Four pieces of roots were placed

on a slide and 50 cells/piece were scored for a total of 200 root cells for each plant.

To determine if mycorrhizal treatment altered plant investment in flowering, I counted inflorescence buds and flowers in each year. I counted the number of overwintering inflorescence buds in March 2019. To control for the varying number of stems per plant, I also counted the total number of stems and those that produced buds to calculate the proportion of branches that formed buds. Additionally, because buds can form along the length of the stem, I measured stem length height and counted the number of primary stems (those growing directly from the soil). Floral data, including the number of inflorescences, number of flowers, and floral measurements, were collected in June 2018 and June 2019. Three flowers per plant were measured in June 2018 and 10 flowers per plant were measured in June 2019. Floral measurements included corolla length, corolla width, and diameter of corolla opening.

To test the phenology between the different treatments, I counted flowers on all plants beginning May 31st, 2019 and counted every 2-3 days until flowering was complete on June 17th, 2019. I recorded the start date of flowering and the date at which each plant had the most flowers in bloom (peak flowering).

To test the hypothesis that inoculation with ericoids alters reproduction through its effects on pollinators, I combined pollinator observations with a hand-pollination experiment throughout the flowering season in 2019. To understand whether the

effects of mycorrhizae on pollinators depend on the pollinator community, 15 plants from each of three treatments (non-inoculated controls, inoculated with commercial ericoid fungi, and inoculated with local soil) were placed at six different farms located in Northeast Vermont known to differ in pollinator abundance and diversity (Nicholson and Ricketts 2019). Plants were observed for 30-minute time blocks, between the hours of 9:00 and 14:00, 3 days per week for the full flowering season from 31 May through 17 June; the time of observation was rotated randomly among treatments and by farm each week I identified each floral visitor as one of the following: queen *Bombus*, worker *Bombus*, orange *Bombus*, *Megachile*, or *Andrenid* (Nicholson et al. 2017, Nicholson and Ricketts 2019). In addition, I recorded the number of flowers visited and the total time spent visiting each plant.

To examine if inoculation affected pollen limitation at each farm, I conducted hand pollination experiments. I assigned each branch to one of two treatment groups: "hand-pollination" in which I artificially added pollen to stigmas, or "open-pollination" in which I allowed plants to be pollinated naturally. During blueberry bloom, I visited the farms every 2-3 days in order to implement the hand-pollination treatment. Pollen

was gathered using a VegiBeeTM miniature sonicator to imitate buzz pollination and release pollen grains. Pollen was collected from a variety of cultivars of blueberry to imitate natural bee foraging behavior and was not collected from experimental

potted plants. Pollen was collected on petri dishes and a paintbrush was used to apply pollen to stigmas of flowers on hand-pollinated treatment branches.

To examine whether inoculation and hand-pollination enhanced fruit characteristics, I counted and collected all berries when ripe. I counted all berries I collected and counted the number of aborted fruits. Average berry mass, berry sugar content, and fertilized seed number were assessed for five berries/plant in 2018 and five berries/branch in 2019. The number of berries collected represented more than 50% of all berries produced by most plants. Seeds were counted using a dissecting scope. Seeds were placed into two categories; small, translucent seeds which appear to be unfertilized or aborted, and fully formed seeds.

1.2.1. Statistical Analysis

To examine if inoculation had an effect on average proportion of roots colonized, an analysis of variance (ANOVA) was used to test for effects of inoculation and date on colonization. Prior to the analysis, the proportion of roots colonized by ericoids was arcsine, square-root transformed, to normalize the data. All statistical analyses were carried out in R 2.9.0 (R Core Team, 2019).

I also used an ANOVA to examine if inoculation had an effect on total inflorescence bud production. Additionally, I analyzed the number of inflorescence buds/total number of stems by mycorrhizal treatment. Year was not included due to stem data only being collected in 2019. Finally, to account for possible differences in available space for buds to form, I calculated plant "volume" by multiplying the number of primary stems by the height. I used an analysis of covariance (ANCOVA) to examine the amount of inflorescence buds formed in 2019. Mycorrhizal treatment was used as a main effect and volume was used as a covariate in the analysis.

To test if there were differences in phenology, the start date of flowering and peak flowering (day at which most flowers were in bloom) were used as dependent variables in a two-way ANOVA with farm and treatment as main effects.

To examine how the interactions of floral visitors differed among treatments, a linear mixed effects model was used. Farm and mycorrhizal treatment were

used as main effects. The response variables used were the number of visits per flower per plant and the total number of seconds a floral visitor remained on a flower during a visit.

To calculate the number of total flowers formed per plant, I took the sum of the fully formed berries and the aborted fruits. To correlate the number of inflorescence buds formed to flowers formed, I used a Spearman's correlation test and calculated the correlation coefficient for inflorescence buds and flowers for each treatment.

To examine if inoculation, hand-pollination, and pollinator context (farm) altered reproduction, fruit set (the percentage of flowers that produced berries) was used as a dependent variable in a linear mixed effects model. Inoculation treatment, hand-pollination treatment, and farm were included as fixed effects. Their interaction effects were also analyzed but were found to be non-significant. To increase the normality of the data, fruit-set was transformed using a log transformation before completing the analysis.

1.3. Results

Prior to inoculation, an average of 0.092 ± 0.013 root cells were colonized by ericoid fungi and plants among treatments did not differ in colonization ($F_{4,71}$ = 1.258; P = 0.295; Table 1; Figure 1). The first collection date, post inoculation, showed that an average of 0.181 ± 0.046 root cells were colonized by ericoids in the non-inoculated control group and 0.328 ± 0.059 were colonized in the commercial inoculum group (Table 2). However, when all collection dates were analyzed, neither inoculation treatment type, nor time, had a significant effect on mycorrhizal colonization ($F_{3,361}$ = 2.569; P = 0.054; $F_{3,361}$ = 1.051; P = 0.370; Table 3).

However, inoculation ($F_{4,607}$ = 11.075; P < 0.001; Table 4) and year ($F_{1,607}$ = 24.594; P < 0.001; Table 4) both had significant effects on the number of inflorescence buds formed. Plant volume did not have a significant effect on the number of total inflorescence buds ($F_{1,607}$ = 2.040; P = 0.154; Table 4). In addition, inoculation had a significant effect on the proportion of branches that produced buds ($F_{3,359}$ = 13.11; P < 0.001; Table 5). On average, plants produced roughly 30% more inflorescence buds in 2019 than in 2018 (Table 6) and inoculated plants produced more inflorescence buds than non-inoculated control plants (Table 6). In 2018, inoculated plants produced, on average, 30% more inflorescence buds than non-inoculated control plants (Table 6). Plants produced more buds if they were inoculated with the combination soil inoculum than the other inoculated treatments or the non-inoculated control plants (Fig 2; Table 6).

Flowering took place from May 31st to June 17th. While there was significant effect of farm on start flowering ($F_{4,468} = 9.696$; $P < 0.001$; Table 7), there was no effect of inoculation treatment on start flowering date ($F_{2,468} = 0.453$; $P = 0.636$; Table 7). Farm also had a significant effect on peak flowering date ($F_{5,571} = 1252$; $P < 0.001$; Table 8), but inoculation did not ($F_{2,571} = 16.64$, $P = 0.287$; Table 8).

Although flowering phenology were the same between treatments, the number of plants that flowered varied significantly between years ($F_{1,759} = 91.009$; $P < 0.0001$; Table 9) and between inoculation treatment ($F_{4,759} = 7.595$; $P < 0.0001$; Table 9). In 2018, 211 plants flowered, but only 122 plants flowered in 2019 out of the 380 plants in total. In 2018, significantly more plants treated with the local soil inoculum flowered (67%; Table 10) than other treatments, while the least number of flowering plants occurred in the non-inoculated controls (45%; Table 10). On average, the inoculated treatments had 15% more flowering plants than the non-inoculated controls (Table 10). Fewer plants bloomed 2019 than in 2018 (31.1 % vs 55.6 % over all treatments) but, again, more plants treated with the local soil inoculum bloomed than in other treatments (Table 10). Moreover, the local soil inoculum treatment showed only a 15% decrease in the number of flowering plants 15% from the previous year, while the number of flowering plants in the commercial inoculum treatment decreased ca. 50% from the previous year (Table 10).

Despite differences in flowering plants, neither inoculation treatment nor farm significantly affected the number of visits per flower a plant received ($F_{2,29}$ = 0.736; P = 0.488; Table 11; $F_{5,29}$ = 1.513; P = 0.216; Table 11). In addition, inoculation did not affect the amount of time a floral visitor spent on a flower ($F_{2,29}$ = 1.563; P = 0.227; Table 12) and neither did farm ($F_{2,29}$ = 0.766; P = 0.582; Table 12).

Fruit set (the proportion of flowers that set fruit) was not significantly affected by hand pollination, ($F_{2,254}$ = 0.784; P = 0.46; Table 14), farm ($F_{2,254}$ = 2.08; P = 0.07; Table 14), mycorrhizal treatment ($F_{5,254}$ = 0.1.475; P = 0.23; Table 14). None of the interactions between hand-pollination, farm, and mycorrhizal treatment were significant

Mycorrhizal treatment and year had significant effects on berry traits including average mass, brix, and number of fertilized seeds. The average individual berry mass was 1.663 ± 0.024 in 2018 and 1.379 ± 0.036 in 2019. There was a significant treatment ($F_{3,323}$ = 4.147; P = 0.007; Table 15) and year ($F_{1,323}$ = 44.734; P < 0.001; Table 15) effect on berry mass. The interaction between treatment and year was not a significant effect on berry mass ($F_{3,323}$ = 1.193; P = 0.313; Table 15).

The average brix content per berry was 12.616 ± 0.173 in 2018 and 11.706 ± 0.178 in 2019. There was a significant treatment effect ($F_{3,323}$ = 4.349; P = 0.005; Table 15), year effect ($F_{1,323}$ = 10.456; P = 0.001; Table 15), and interaction of treatment and year effect ($F_{3,323}$ = 4.456; P = 0.004; Table 15) on berry brix level.

The average number of fertilized seeds per berry was 70.744 ± 0.927 in 2018 and 65.348 ± 1.592 in 2019. There were significant treatment ($F_{3,323}$ = 3.306; P = 0.021; Table 15) and year ($F_{1,323}$ = 8.417; P = 0.004; Table 15) effects on the number of fertilized seeds per berry. The interaction effect between treatment and year was not significant ($F_{3,323}$ = 2.524; P = 0.058).

1.4. Discussion

Plants often interact with mycorrhizal fungi and animal pollinators simultaneously. Here, I found that inoculation with ericoid mycorrhizal fungi directly affected floral traits but there was no effect on pollinator visitation between the treatments. The effects of inoculation varied with the type of inoculum used and the time since inoculation.

Despite low numbers of flowering plants and similar levels of root colonization by ericoids in the commercial and local soil inoculum treatments, the number of plants that flowered in the local soil inoculum treatment was almost double that of plants that flowered in the other treatments in the flowering season of 2019. In addition, I found that there was no detectable correlation between the number of buds that the plants produced in the fall of 2018 and the number of flowers produced in the 2019 for any of the treatments. The most likely cause for this was that the plants were infected by two fungal diseases, *Fusicoccum putrefaciens* and *Phomopsis vaccinii*, during the spring of 2019. These diseases could have altered the number of plants that flowered in each treatment after buds had already been preformed and weakened the links between the mycorrhizal treatment and aboveground traits and interactions.

It was a cool and wet spring which are ideal conditions for certain fungal diseases like *F. putrefaciens* and *P. vaccinii* to spread (Parker and Ramsdell 1977). Although virtually all plants showed signs of disease, and there were no correlations detected between buds and flowers for any of the treatments, the mycorrhizal treatments seemed to alter the effects that the disease had on the plants. The proportion of flowering plants in the commercial inoculum dropped 34% from 2018 to 2019 (Table 10) which is more drastically than the other inoculated treatments (combination soil 24%; local soil 17%; Table 10). This would support the idea that the disease most negatively affected the plants inoculated with commercial inoculum.

Associating with mycorrhizal fungi can downregulate plant defense pathways (Fouad et al. 2014, Benhiba et al. 2015) and therefore leave a plant more vulnerable to diseases. Thus, it is possible that plants in the commercial inoculum could have been more damaged by disease than other treatments because of a lowered immune system. Then one would expect that the non-inoculated controls would have performed the best followed by the local soil, the combination soil, and the commercial inoculum. My results show that, the local soil inoculum had the most flowering plants in 2019 – 24% more flowering plants than the non-inoculated control. However, the disease decreased the local soil treatment and the non-inoculated controls by a very similar amount (15% and 17% decrease, respectively) followed by the combination soil and commercial inoculum as expected.

Arbuscular mycorrhizal fungi has been shown to offer protection from disease in many cases (Bizos et al. 2020, Gao et al. 2020, Kadam et al. 2020). It is possible that local soils may be inhabited by fungi adapted to Vermont conditions and, therefore, better mutualists in providing protection to plants. The non-inoculated control and local soils are both Vermont based soils. They may be more adapted to defend against common soil pathogens such as those that infected my plants in 2019. Examining the genetic sequences of these fungi would help form connections between taxa of below- and aboveground fungal taxa. Further work needs to be done to understand the interactions between mutualistic fungi and pathogenic fungi.

The average number of inflorescence buds per plant varied between treatments and years. Some patterns held true in both years such as the non-inoculated control had the least amount of inflorescence buds each year they were counted. However, in 2018, plants treated with local soil inoculum produced significantly more buds than those in the other treatments, while in 2019, plants treated with the combination soil treatment produced more buds than those in the other treatments. The fungal species found in the roots of the combination soil plants may be more diverse than the others. The differences between the fungal species could translate into access to different benefits for the host plant. Arbuscular mycorrhizal fungi has been shown to provide different benefits to different host plants by discriminating against more mutualistic or more parasitic plant hosts (Kiers et al. 2011).

While ericoids generally help their host uptake nitrogen (Read 1991), it is also possible that not all ericoids are equal in their ability to extract N from different sources and provide it to their plant hosts. For example, ericoid species vary in the rates at which they absorb ammonium and nitrate (Midgley et al. 2004); thus, the presence of multiple species of ericoids may increase nitrogen uptake. Ericoids can also use chitin as a nitrogen source (Leake and Read 1990), but it is likely that only some species of ericoids have this ability.

Association with arbuscular mycorrhizal fungi can enhance investment in flowers and floral rewards and increase attractiveness to pollinators (Gange and Smith 2005). I expected that the association with ericoid mycorrhizal fungi would act similarly. Additionally, I expected the increase in attractiveness to pollinators to increase fruit and seed set. Increase in floral display can increase pollination and lead to higher reproductive success (Karron and Mitchell 2012). Because of this, I specifically, expected that the number of plants in bloom would attract more pollinators to the local soil treatment. I also expected to see pollinators remain on inoculated plants for a longer time due to more attractive floral display. However, there was no significant difference in the time pollinators spent per plant among treatments. Although floral display can be important to pollinators and treatment affected floral display, there could be other floral traits that are important to pollinators. These may include pollen or nectar levels, which I did not measure. *Vaccinium corymbosum* receives more visits with a higher

abundance of nectar (Jablonski et al. 1985). Associating with mycorrhizal fungi might lower the level of sugar plants add to their nectar due to the amount of carbohydrates they need to donate to their fungal partner (Becklin et al. 2011). Colonization with mycorrhizal fungi may also decrease floral volatiles that attract pollinators hence leading to a decrease in visits (Becklin et al. 2011). It is also possible that I did not have sufficient power to detect differences among treatments as few of the plants in all treatments bloomed and visit numbers to these plants were low overall.

Vaccinium corymbosum is often pollen-limited (Dogterom et al. 2000, Nicholson and Ricketts 2019) and, therefore, I expected hand pollination would increase fruit set, at least at some farms. I specifically chose farms that differed in abundance of pollinators (Nicholson and Ricketts 2019) and gathered pollen from a mix of blueberry plants and cultivars because outcrossed or mixed pollen is more effective than self-pollination for Bluecrop (Dogterom et al. 2000). It is important to consider that the different farms had different numbers of each cultivar and therefore the mix of pollen was different at each farm. However, I did not find evidence for pollen limitation. There are several potential reasons for this finding. First, the degree of pollen limitation varies among years for many plants (Knight et al. 2005). Second, it is possible that I inadvertently damaged stigmas or caused clogging of stigmas by using an abundance of incompatible pollen (Ashman et al. 2004). However, it's most parsimonious to conclude that my plants got sufficient levels of bee visits such that most flowers produced fruits regardless of whether

they were hand-pollinated or not and they were not pollen limited.

Plant reproduction is often nutrient limited (Claussen and Lenz 1995, Morrison and Questad 2019, Strik et al. 2019, Pers-Kamczyc et al. 2020) or pollinator limited (Drummond 2019). Given that I saw no differences in pollinator visitation, I expected that nutrients would be the limiting factor for reproduction. Blueberry reproduction can be specifically nitrogen limited (Ehret et al. 2014, Strik et al. 2019) and because ericoids enhance nitrogen uptake (Read 1991), it was expected that inoculation would increase fruit set and number of fertilized seeds produced per berry. I also expected to see an increase in fruit set and number of fertilized seeds per berry when compared to 2018 due to plants having a stronger relationship with their fungal partners. However, I did not see evidence of plants having a stronger relationship with mycorrhizal fungi in 2019 based on the proportion of cells colonized by ericoids. In addition, saw no difference in fruit set between the treatments and I saw lower numbers of fertilized seeds per berry and berry mass in 2019. It is possible that disease weakened the links between inoculation and the aboveground traits such as berry production. Plants are likely to have expended resources defending against and recovering from infection and thus had fewer resources for reproduction. It is also possible that highbush blueberry faces tradeoffs in associating with mycorrhizal fungi. A large amount of carbon is allocated towards microbial relationships, such as mycorrhizae, in perennial fruit crops (Buwalda 1993). When involved in a symbiosis, it is also common to encounter cheats who take more resources

than they provide (Douglas 2008, Kiers et al. 2011). Associating with mycorrhizal fungi also decreases defense responses in agricultural crops, such as *Medicago sativa* (Kapulnik et al. 1996). Any or all of these costs could have affected my plants causing fewer fertilized seeds per berry in 2019.

Several caveats must be considered in interpreting our results. First of all, mycorrhizal fungi can range from parasitic to mutualistic (Klironomos 2003) therefore, greater infectivity by fungi does not guarantee increased benefits to the plant. Although the commercial inoculum is a general inoculum for plants within the Ericaceae, the local soil inoculum was taken from the rhizosphere of plants of the same cultivar used in my study. The spores in this soil are likely more compatible with the BlueCrop host I used, however, the presence of different taxa need to be with DNA sequencing. Learning the taxonomic identity of these fungi will confirm differences between fungal communities. Spore germination is important in establishment and persistence of fungi which can be linked to many environmental factors such as temperature and moisture level (de Novais et al. 2013, Giovannini et al. 2020). Although my experimental plants and fungi are from different areas, the local soil inoculum may be more successful in Vermont conditions than the commercial inoculum used because it has adapted to Vermont conditions. This could lead to a more effective inoculum.

Second, the disease that the plants endured during the 2019 field season affected the health of my plants and traits they displayed. The disease most likely weakened the effects of inoculation of many of the interactions studied. In addition, the drastic decrease in flowering plants during 2019 led to lower sample sizes while studying floral traits and interactions with floral visitors making it difficult to detect differences between treatments.

Lastly, I did not see strong differences in colonization of cortical cells by ericoids. This is most likely due to the high variability of ericoids within roots. Although the amount of ericoids present may not be different between treatments, it is possible that the taxa present in the roots is different between treatments. This is likely as all was held constant between my plant treatments with the exception of whether or not they were inoculated and by which inoculum. However, I did see differences in plant traits such as number of inflorescence buds and the proportion of plants that flowered.

My results, demonstrate that the relationships between highbush blueberry and its ericoid, mycorrhizal fungi are complex. Many factors influence this relationship including, time post inoculation, life stage of plant, type of inoculum, and interactions with fungal pathogens. It appears that ericoid, mycorrhizal partners can directly enhance reproductive traits in *V. corymbosum*, but fungal genotypes should be examined to get a fuller understanding of the relationship. Future molecular work that aims to identify the

genetic components of the different strains on fungi present will significantly advance the knowledge in this field. In addition, pollinator visitation and floral rewards need to be studied more thoroughly to understand how mycorrhizal fungi can affect interactions with pollinators. In addition, the links between ericoids and aboveground interactions, such as between plants and their fungal pathogens, need to be researched in a more controlled setting. My research here increases the knowledge of how belowground interactions can directly affect aboveground plant traits and reveals more questions for future studies.

1.5. Figures

1.5.1 Tables

Table 1 Analysis of variance showing root colonization by ericoid mycorrhizal fungi prior to inoculation in Spring 2018. The proportion of root cells colonized was arcsine-square root transformed prior to analysis. All five treatment groups were used in analysis. Treatment (MycTrt) was not a significant effect on colonization ($F_{4,71}$ = 0.046; P = 0.295).

	Df	*Sum Sq*	*Mean Sq*	*F value*	*Pr(>F)*
MycTrt	4	0.1856	0.0464	1.258	0.295
Residuals	71	2.6198	0.0369		

Table 2 Mean (1 ± std. error) proportion of root cells in which an ericoid hyphal coil was found per plant in the non-inoculated control, the commercial inoculum, the combination soil and the local soil treatments. Data shown is prior to inoculation and post inoculation at three additional collection dates in 2018 and 2019. There was no significant effect of treatment ($F_{3,361}$ = 2.569; P = 0.054) or date ($F_{3,361}$ = 1.051; P = 0.370) on root colonization by ericoids.

	Prior to Inoculation	*18-Sep*	*19-Jun*	*19-Sep*
No Inoculum	0.130 ± 0.028	0.181 ± 0.046	0.351 ± 0.040	0.278 ± 0.039
Commercial Inoc.	0.136 ± 0.039	0.328 ± 0.059	0.412 ± 0.038	0.308 ± 0.032
Combination Soil	0.091 ± 0.027	0.229 ± 0.050	0.308 ± 0.035	0.333 ± 0.036
Local Soil	0.113 ± 0.032	0.147 ± 0.036	0.274 ± 0.029	0.324 ± 0.031

Table 3 Proportion of root cells colonized as a function of date and inoculation treatment (MycTrt). The proportion of root cells colonized were arcsine-square root transformed prior to analysis. Non inoculated control, commercial inoculum, combination soil, and local soil treatments were included in analysis. Neither treatment ($F_{3,361}$ = 2.57; P = 0.05) nor date ($F_{3,361}$ = 1.05; P = 0.37) had a significant effect on root colonization.

	Df	*Sum Sq*	*Mean Sq*	*F value*	*Pr(>F)*
MycTrt	3	0.414	0.138	2.569	0.054
Date	3	0.170	0.057	1.051	0.370
Residuals	361	19.416	0.054		

Table 4 Analysis of variance table (Type III tests) examining effects of treatment (MycTrt) and year on total inflorescence buds per plant with plant volume (number of primary stems * average height of stems) as a covariate. The test examined plants in all five treatment groups in 2018 and 2019. Treatment had a significant effect on the number of total inflorescence buds per plant ($F_{4,607}$ = 11.075; P < 0.001) as did year ($F_{1,607}$ = 24.594; P < 0.001) while volume did not ($F_{1,607}$ = 2.040; P = 0.154).

	Sum Sq	*Df*	*F value*	*Pr(>F)*
(Intercept)	8588	1	24.538	**< 0.001 ***
MycTrt	15504	4	11.075	**< 0.001 ***
Year	8607	1	24.594	**< 0.001 ***
Volume	714	1	2.040	0.154
Residuals	212437	607		

Table 5 Analysis of variance table testing treatment (MycTrt) and year effects on the proportion of branches that produced buds per plant for non-inoculated control, commercial inoculum, combination soil, and local soil for 2019. Data was arcsine-square root transformed prior to analysis. Treatment had a significant effect on the proportion of branches that produced buds ($F_{3,359}$ = 13.11; P < 0.001).

	Df	*SumSq*	*MeanSq*	*F Value*	*Pr(>F)*
MycTrt	3	1.432	0.4774	13.11	**< 0.001 ***
Residuals	359	13.072	0.0364		

Table 6 Mean (± 1 std. error) number of total inflorescence buds per plant in 2018 and 2019 and the proportion of branches that produced buds per plant in 2019 in each of the five treatments. There was a significant effect of treatment ($F_{4,629}$ = 10.64; P < 0.001) and year ($F_{1,629}$ = 45.52; P < 0.001) on the number of total inflorescence buds per plant. There was a significant effect of treatment on the proportion of branches producing buds ($F_{3,359}$ = 13.11; P < 0.001). Results were analyzed using a two-way analysis of variance test and then a post-hoc Tukey HSD test to examine the differences between treatments; different letter represent significant differences.

	Total inflorescence buds		*proportion of branches producing buds*
	2018	**2019**	**2019**
No Inoculum	23.189 ± 1.396 **b**	34.879 ± 2.590 **ac**	0.320 ± 0.017 **a**
Peat Control	23.889 ± 3.239 **ab**	40.750 ± 6.470 **acd**	--
Commercial Inoculum	31.156 ± 1.640 **abc**	39.650 ± 2.891 **acd**	0.415 ± 0.019 **b**
Combination Inoculum	36.730 ± 1.760 **ac**	49.433 ± 3.235 **d**	0.457 ± 0.019 **b**
Local Soil	33.967 ± 1.422 **ac**	40.967 ± 3.395 **cd**	0.467 ± 0.017 **b**

Table 7 Analysis of variance table examining the start flowering date with treatment (MycTrt) and farm as main effects. Analysis done for plants in the non-inoculated control, the commercial inoculum treatment, and the local soil treatment at different farms. There was no effect of treatment on starting flowering date ($F_{2,468}$ = 0.453; P = 0.636); but the farm did have an effect on the start flowering date ($F_{5,468}$ = 9.696; P < 0.001).

	Df	*Sum Sq*	*Mean Sq*	*F value*	*Pr(>F)*
MycTrt	2	12	5.98	0.453	0.636
Farm	5	639	127.9	9.696	**< 0.001 ***
Residuals	468	6173	13.19		

Table 8 Analysis of variance table examining peak flowering date with treatment (MycTrt) and farm as main effects. Analysis done for plants in the non-inoculated control, the commercial inoculum treatment, and the local soil treatment at different farms. There was no effect of treatment on peak flowering date ($F_{3,571}$ = 1252; P = 0.287); but the farm did have an effect on the peak flowering date ($F_{5,571}$ = 10.104; P < 0.001).

	Df	*Sum Sq*	*Mean Sq*	*F value*	*Pr(>F)*
MycTrt	2	33	16.64	1252	0.287
Farm	5	671	134.25	10.104	**< 0.001 ***
Residuals	571	7587	13.29		

Table 9 Analysis of variance table testing treatment (MycTrt) and year effects on the number of plants that flowered in 2018 and 2019 in each of the five treatments. Both treatment ($F_{4,759} = 7.595$; $P < 0.001$) and year ($F_{1,759} = 91.009$; $P < 0.001$) had an effect on the number of plants that flowered.

	Df	*Sum Sq*	*Mean Sq*	*F Value*	*Pr(>F)*
MycTrt	4	6.59	1.648	7.595	**< 0.001 ***
Year	1	19.74	19.742	91.009	**< 0.001 ***
Residuals	759	164.64	0.217		

Table 10 The proportion of plants that flowered out of all plants in 2018 and 2019 in each of the five treatments. Both treatment ($F_{4,759} = 7.595$; $P < 0.001$) and year ($F_{1,759} = 91.009$; $P < 0.001$) had an effect on the number of plants that flowered.

	Proportion of plants that flowered	
Year	**2018**	**2019**
No Inoculum	0.45	0.28
Peat Control	0.50	0.05
Commercial Inoculum	0.57	0.23
Combination Inoculum	0.54	0.30
Local Soil	0.67	0.52

Table 11 The number of floral visits per flower per plant in the non-inoculated control, commercial inoculum treatment, and the local soil treatment in 2019 as a function of farm and inoculation. There were no significant effects of farm ($F_{5,29}$ = 1.513; P = 0.216) or treatment ($F_{2,29}$ = 0.736; P = 0.488) on the number of floral visitors per flower per plant.

	Df	*SumSq*	*MeanSq*	*Fvalue*	*Pr(>F)*
Farm	5	1.311	0.262	1.513	0.216
MycTrt	2	0.255	0.128	0.736	0.488
Residuals	29	5.026	0.173		

Table 12 The time a floral visitor spent per flower in the non-inoculated control, commercial inoculum treatment, and the local soil treatment in 2019 as a function of farm and mycorrhizal treatment. There were no significant effects of farm ($F_{5,29}$ = 0.766; P = 0.582) or treatment ($F_{2,29}$ = 1.563; P = 0.227) on the time a floral visitor spent per flower on any of the test treatments.

	Df	*SumSq*	*MeanSq*	*Fvalue*	*Pr(>F)*
Farm	5	2959	591.8	0.766	0.582
MycTrt	2	2416	1208.2	1.563	0.227
Residuals	29	22418	773		

Table 13 Fruit set in 2019 as a function of hand pollination treatment (HpTrt), farm, and inoculation treatment (MycTrt). None of the main effects including hand pollination treatment ($F_{2,254}$ = 0.784; P = 0.457), farm ($F_{5,254}$ = 2.083; P = 0.068), and inoculation treatment ($F_{2,254}$ = 1.475; P = 0.231) were found to have significant effects on fruit set as a response variable.

	Df	*SumSq*	*MeanSq*	*Fvalue*	*Pr(>F)*
HpTrt	2	0.009	0.005	0.784	0.457
Farm	5	0.061	0.012	2.083	0.068
MycTrt	2	0.017	0.009	1.475	0.231
Residuals	254	1.480	0.006		

Table 14 Analysis of variance table for effects of treatment (MycTrt), year, and interaction effect between treatment and year on individual berry mass, sugar content, and total fertilized seed count. Treatment ($F_{3,323}$ = 4.147; P = 0.007) and year ($F_{3,323}$ = 44.734; P < 0.001) had significant effects on berry mass, while their interaction did not ($F_{3,323}$ = 1.193; P = 0.313). Treatment ($F_{3,323}$ = 4.349; P = 0.005), year ($F_{1,323}$ = 10.456; P = 0.001), and their interaction ($F_{3,323}$ =4.456; P = 0.004) had significant effects on individual berry sugar content (Brix). Treatment ($F_{3,323}$ = 3.306; P = 0.021) and year ($F_{1,323}$ = 8.417; P = 0.004) had significant effects on berry mass, while their interaction did not ($F_{3,323}$ = 2.524; P = 0.058).

Mass	Df	Sum Sq	Mean Sq	F value	Pr(>F)
MycTrt	3	1.63	0.543	4.147	**0.007 ***
Year	1	5.86	5.846	44.734	**< 0.001 ***
*MycTrt * Year*	3	0.47	0.156	1.193	0.313
Residuals	323	42.28	0.131		
Brix	Df	Sum Sq	Mean Sq	F value	Pr(>F)
MycTrt	3	65.2	21.74	4.349	**0.005 ***
Year	1	52.3	52.26	10.456	**0.001 ***
MycTrt Year*	3	66.9	22.31	4.456	**0.004 ***
Residuals	323	1614.3	5.00		
Total Fertilized Seeds	Df	Sum Sq	Mean Sq	F value	Pr(>F)
MycTrt	3	2258	752.7	3.306	**0.021 ***
Year	1	1916	1916.5	8.417	**0.004 ***
*MycTrt * Year*	3	1724	574.7	2.524	0.058
Residuals	323	73542	227.7		

Table 15 Mean (± 1 std. error) berry mass, sugar content (Brix), and total fertilized seed count from 2018 and 2019 from non-inoculated control, commercial inoculum, combination soil, and local soil. Results were analyzed using a two-way analysis of variance test and then a post-hoc Tukey HSD test to examine the differences between treatments; different letter represent significant differences.

	No Inoculum		*Commercial Inoculum*		*Combination Soil*		*Local Soil*	
Year	*2018*	*2019*	*2018*	*2019*	*2018*	*2019*	*2018*	*2019*
Mass ± SE	1.622 ± 0.057 ab	1.466 ± 0.064 bc	1.800 ± 0.039 a	1.436 ± 0.098 bc	1.616 ± 0.045 ab	1.349 ± 0.079 c	1.613 ± 0.046 ab	1.309 ± 0.056 c
Brix ± SE	12.415 ± 0.433 ab	11.322 ± 0.413 b	13.223 ± 0.362 a	11.581 ± 0.411 ab	12.440 ± 0.275 ab	13.289 ± 0.363 a	12.367 ± 0.318 ab	11.109 ± 0.225 b
Total Fertilized Seeds ± SE	67.057 ± 1.864 ab	69.455 ± 2.906 ab	76.553 ± 1.707 a	66.229 ± 4.536 ab	70.200 ± 1.979 ab	60.959 ± 4.200 b	68.611 ± 1.654 ab	65.188 ± 2.110 b

1.5.2 Figures

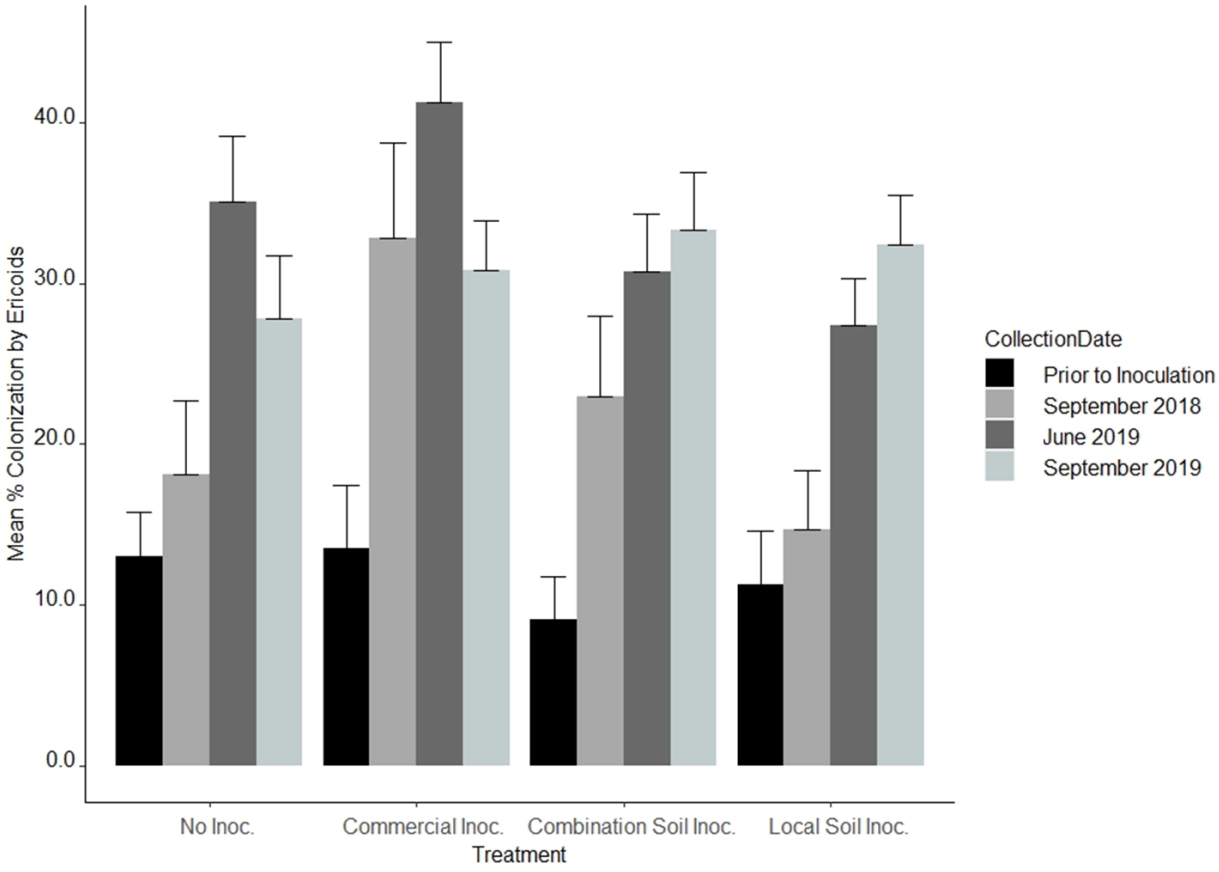

Figure 1 The mean (± 1 std error) percentage of root cells in which an ericoid hyphal coil was found per plant in each of the five treatments in 2018-2019. Raw percentages are shown for clarity, however all values were arcsine-square root transformed prior to analysis. There was no significant effect of treatment ($F_{4,384}$=2.184, P = 0.070) or date ($F_{3,384}$=1.666, P = 0.174) on colonization. N = 15 plants/treatment prior to inoculation and N=30 plants/treatment for all other collection dates except for the Peat Control where N=10 plants

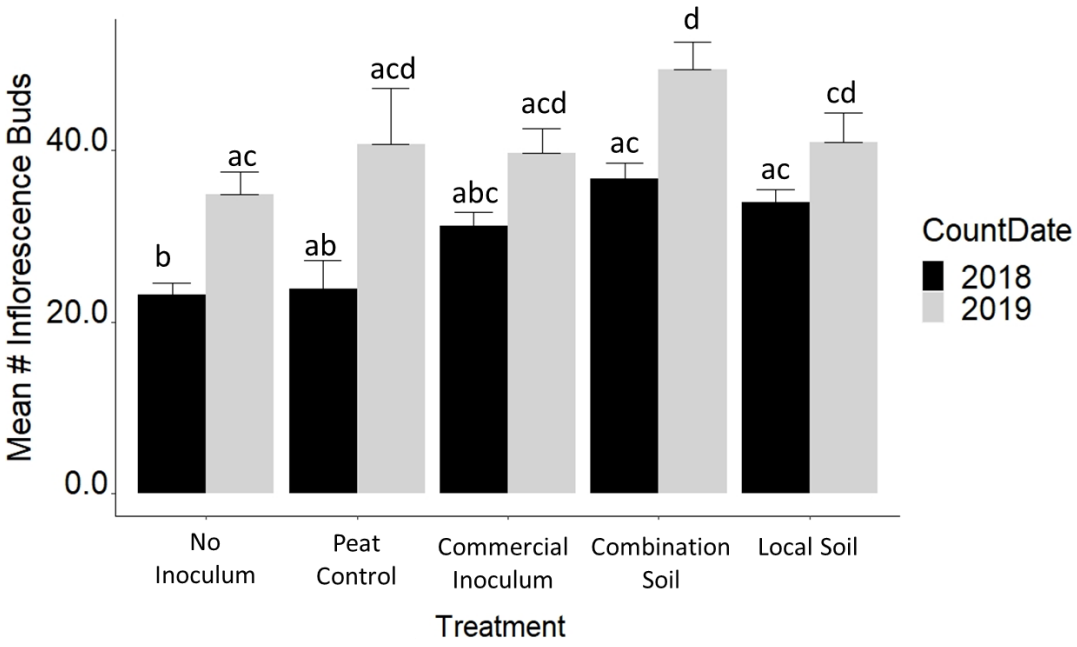

Figure 2 The mean (±1 std error) number of inflorescence buds formed per plant in each of the five treatments in 2018-2019. Results were analyzed with a two-way analysis of variance which showed that treatment ($F_{4,629}$ = 10.64; P < 0.001) and year ($F_{1,629}$ = 45.52; P < 0.001) had significant effects on the number of inflorescence buds per plant. A post-hoc Tukey's HSD test was then used to differentiate between treatment means. Letters denote significant differences.

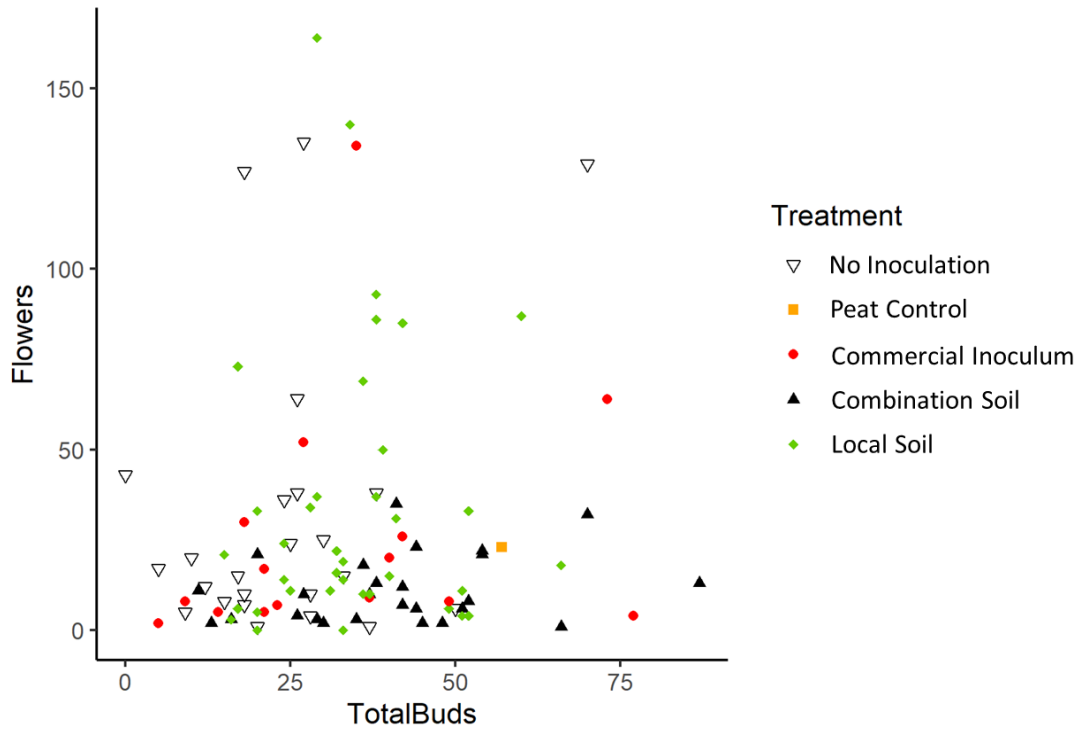

Figure 3 Correlation between the total number of inflorescence buds produced per plant and the total number of flowers produced per plant for each of the five treatments in 2019. There was no identifiable correlation between inflorescence buds and flowers in any treatment. The peat control group did not have enough data in order to calculate the correlation coefficient. The coefficients for the other groups are as follows: Non-inoculated control (R = 0.068; P = 0.752), commercial inoculum (R = 0.296; P = 0.266), combination soil (R = 0.217; P = 0.287), and local soil (R = 0.142; P = 0.402).

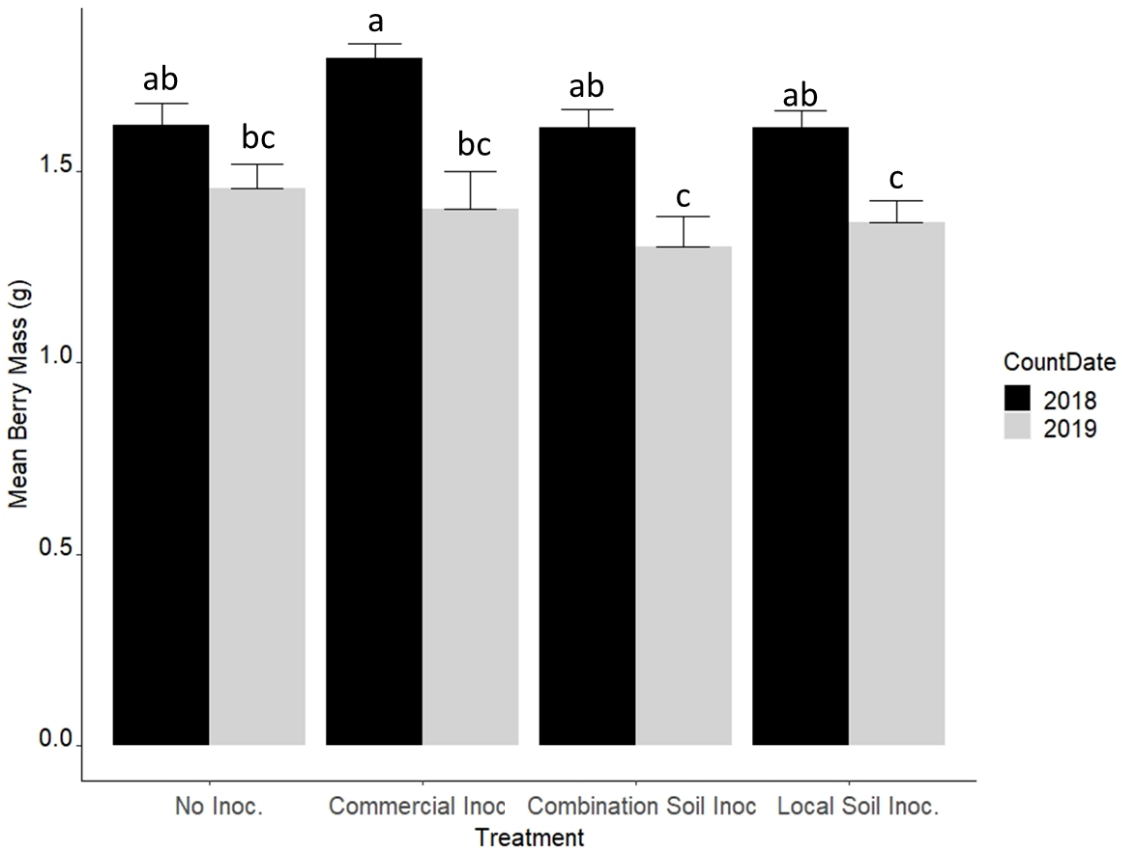

Figure 4 The mean (±1 std error) individual berry mass for the non-inoculated control, commercial inoculum, combination soil, and local soil for 2018-2019. Treatment ($F_{3,323}$ = 4.147; P = 0.007) and year ($F_{3,323}$ = 44.734; P < 0.001) had significant effects on berry mass, while their interaction did not ($F_{3,323}$ = 1.193; P = 0.313). Results were analyzed using a two-way analysis of variance test and then a post-hoc Tukey HSD test to examine the differences between treatments; different letter represent significant differences.

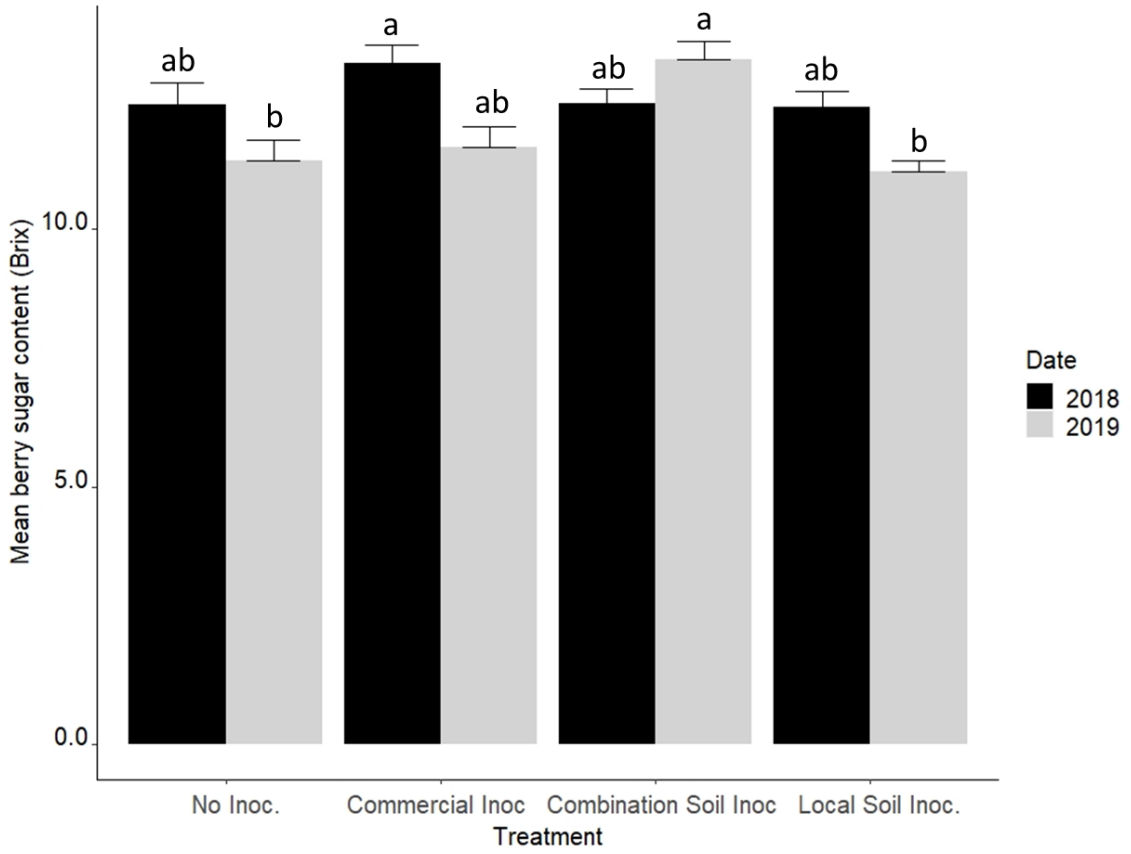

Figure 5 The mean (±1 std error) sugar content per berry for the non-inoculated control, commercial inoculum, combination soil, and local soil for 2018-2019. Treatment ($F_{3,323}$ = 4.349; P = 0.005), year ($F_{1,323}$ = 10.456; P = 0.001), and their interaction ($F_{3,323}$ = 4.456; P = 0.004), all had significant effects on the mean berry sugar content for 2018-2019. Results were analyzed using a two-way analysis of variance test and then a post-hoc Tukey HSD test to examine the differences between treatments; different letter represent significant differences.

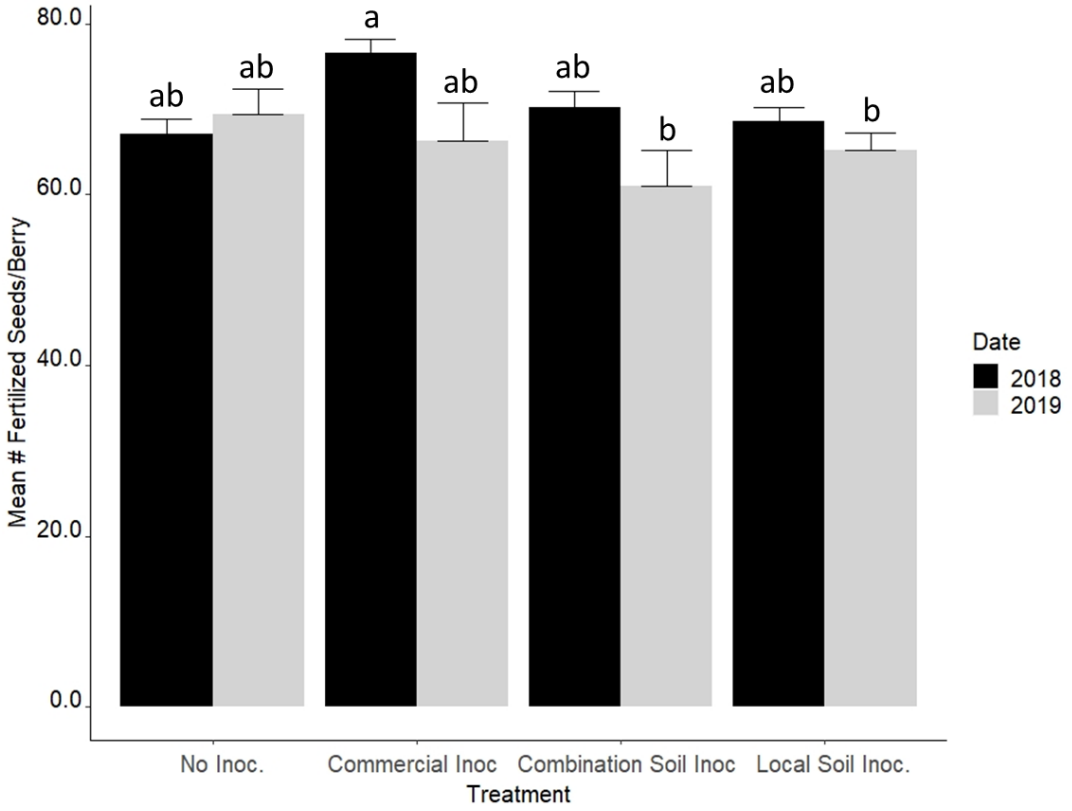

Figure 6 The mean (±1 std error) number of fertilized seeds produced per berry for the non-inoculated control, commercial inoculum, combination soil, and local soil for 2018-2019. Treatment ($F_{3,323}$ = 3.306; P = 0.021) and year ($F_{1,323}$ = 8.417; P = 0.004) had significant effects on the mean number of fertilized seeds for 2018-2019 while their interaction did not ($F_{3,323}$ = 2.524; P = 0.058). Results were analyzed using a two-way analysis of variance test and then a post-hoc Tukey HSD test to examine the differences between treatments; different letter represent significant differences.

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